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Ornithobacterium rhinotracheale North American Field Isolates Express a Hemolysin-Like Protein

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SUMMARY. *Ornithobacterium rhinotracheale* is a gram-negative bacterium responsible for the sporadic outbreaks of airsacculitis in poultry, accounting for millions of dollars in losses to the poultry industry annually. Although the organism was originally classified as non–β-hemolytic, recent North American field isolates of *O. rhinotracheale* obtained from pneumonic lungs and air sacs indicated hemolytic activity on blood agar plates upon extended incubation for 48 hr at room temperature in air after initial incubation at 37 C for 48 hr under 7.5% CO_2. This report characterizes the β-hemolytic activity of *O. rhinotracheale* isolates by using *in vitro* kinetic hemolysis assays with sheep red blood cells, western blotting with leukotoxin-specific monoclonal antibodies, and isobaric tagging and relative and absolute quantitative (iTRAQ) analysis of *O. rhinotracheale* outer membrane protein digest preparations. The kinetic analyses of the hemolytic activity with red blood cells indicated that the protein is a pore former. iTRAQ analysis with membrane preparations revealed four peptides with homology to *Mannheimia haemolytica* leukotoxin and two peptides with homology to *Actinobacillus actinoacetemcomitans* leukotoxin. This is the first report that North American field isolates of *O. rhinotracheale* may express a hemolysin-like activity.

RESUMEN. Los aislamientos de campo de *Ornithobacterium rhinotracheale* de América del Norte expresan una proteina tipo hemolisina.

Ornithobacterium rhinotracheale es una bacteria gram-negativa responsable de brotes esporádicos de aerosaculitis en aves comerciales, lo que representa millones de dólares en pérdidas a la industria avícola por año. Aunque el organismo originalmente fue clasificado como no β-hemolítico, aislamientos recientes de *O. rhinotracheale* de América del Norte, obtenidos de pulmones con neumonía y de sacos aéreos, mostraron actividad hemolítica en placas de agar sangre después de una incubación prolongada durante 48 horas a temperatura ambiente en el aire después de una incubación inicial a 37 C durante 48 horas en un ambiente con 7.5% de CO_2. En este reporte se caracterizó la actividad β-hemolítica de los aislamientos de *O. rhinotracheale* mediante ensayos de la cinética de la hemólisis *in vitro* con eritrocitos de oveja, inmunoelectrotransferencia con anticuerpos monoclonales específicos contra leucotoxina y por los análisis cuantitativos relativos y absolutos de las preparaciones de la digestión de la proteína externa de membrana de *O. rhinotracheale* (con las siglas en inglés iTRAQ). El análisis cinético de la actividad hemolítica con los eritrocitos indicaron que la proteína forma poros. El análisis iTRAQ con preparaciones de membrana reveló cuatro péptidos que mostraban homología con la leucotoxina de *Mannheimia haemolytica* y dos péptidos con homología con la leucotoxina de *Actinobacillus actinoacetemcomitans*. Este es el primer reporte que describe que los aislamientos de campo de *O. rhinotracheale* de América del Norte pueden expresar una actividad similar a la de una hemolisina.

Key words: *Ornithobacterium rhinotracheale*, hemolysin, hemolysis

Abbreviations: BHI = brain-heart infusion; HEPES = 4-(2-hydroxyethyl) piperizane-1-ethanesulfonic acid; iTRAQ = isobaric tagging and relative and absolute quantitative; lktA = leukotoxin A; PBS = phosphate-buffered saline; RBC = red blood cell; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

Ornithobacterium rhinotracheale is a gram-negative emerging pathogen of adult poultry (12) and occurs worldwide (1). In the United States, despite the availability of autogenous vaccines, economic losses to the poultry industry are estimated in hundreds of millions of dollars annually (12). Clinical signs include sinusitis, airsacculitis, arthritis, and increased mortality (10,17,36,38,43) and lowered egg production in turkey layers (28) and laying chickens (44). Poultry can carry *O. rhinotracheale* and seroconvert in the absence of overt clinical signs (3,25). Outbreaks of *O. rhinotracheale* are seasonal and usually occur as a secondary infection (34,51), and often have minimal effect on the outcome of the primary infection (27,36). Outbreaks of *O. rhinotracheale* as the primary agent have also been reported (52).

In the absence of information about the genome sequence it is not possible to query the sequence and identify potential virulence factors for *O. rhinotracheale*, but a few reports describe potential virulence factors. For example, a potential virulence-associated plasmid-encoded protein of unknown function was described (26). Most pathogens are known to express redundant mechanisms of virulence, including iron acquisition mechanisms (14,15,16,24,45), colonization strategies (20,21,33,35,37), and adaptations that alter immune cell function through the toll-like receptor–mediated signaling pathway (7). Some pathogens secrete cytolytic toxins that affect host cell function, such as leukotoxin A (lktA) of *Mannheimia haemolytic*a (32), or the hemolysin HlyA of *Escherichia coli* (18,23). No cytolytic activities have been reported for *O. rhinotracheale*. Indeed, the ATCC 51463 strain of *O. rhinotracheale* was described as non–β-hemolytic (49). We observed, however, that some field strains of *O. rhinotracheale* exhibited typical but weak β-hemolytic

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Table 1. Description of *O. rhinotracheale* isolates used in this study.

Isolate no.	Serotype	Tissue source	Designation/origin
P5883	C	Unknown	Strain 31, University of Minnesota
P5884	A	Unknown	Strain 83, University of Minnesota
P5886	E	Unknown	Strain 87, University of Minnesota
P5887	A	Lung (pneumonia)	Strain 88, University of Minnesota
ATTC strain 51463	A	Respiratory tract	ATCC strain 51463, U.K.
Isolates capable of growth under iron-restricted conditions[A]			
P5902		Lung	3311-05/AR
P5917		Unknown[B]	05-573/CA
P5921		Unknown[B]	Ko500325/CA
P5932		Lung	H06-030791/NC
P5938		Lung	06-Jo/NC
P5945		Lung	D05-045749/MN
P5952		Liver	D05-062700/MN
P5967		Unknown	D06-10652/MN
P5969		Unknown	D06-12314/MN
Isolates incapable of growth under iron-restricted conditions[A]			
P5907		Trachea	167-06/MO
P5908		Lung	1012-06/CO
P5909		Trachea	1513-06/AR
P5913		Not known[B]	K02-181/CA
P5926		Not known[B]	97-7091/GA
P5936		Lungs	R06-025944/NC
P5940		Trachea	D05-043244/MN
P5963		Unknown[B]	D06-30480/MN
P5970		Unknown[B]	D0611105/MN

[A]Cultures grew in the presence and absence of 100 μM 2,2′-dipyridyl (see reference 41).
[B]Data not available.

reactions on sheep blood agar plates (46) similar to what has been observed for *M. haemolytica* (8). Depending on the strain used, development of β-hemolytic activity of *O. rhinotracheale* was a delayed reaction and was often observed after 48 hr of incubation under 7.5% CO_2 followed by 48 hr of aerobic incubation at room temperature.

The objective of this work was to compare and characterize biochemically and kinetically the hemolysin-like activity of *O. rhinotracheale* field isolates.

MATERIALS AND METHODS

Cultures and growth conditions. *Ornithobacterium rhinotracheale* used in this study were turkey isolates and are summarized in Table 1. Stock cultures were stored at −80 C in brain-heart infusion (BHI) broth (Becton-Dickinson, Sparks, MD) containing 20% glycerol. Isolates from our culture collection were previously selected for their ability or inability to grow in the presence of 100 μM 2,2′-dipyridyl (45) to stimulate iron acquisition mechanisms.

For assays of hemolytic activity on blood agar plates, a loop-full of stock culture was streaked on a trypticase soy agar plate containing 5% defibrinated sheep blood (Becton-Dickinson) and incubated (48 hr at 37 C, 7.5% CO_2, 15% relative humidity). Evidence for both α-hemolysis (greening of the blood agar under the confluent growth, but no clearing of the blood agar) and β-hemolysis (partial to complete clearing of the blood agar under confluent growth and individual colonies) was recorded daily.

For *in vitro* hemolytic assays, 10 colonies were removed and transferred to a 10 ml culture tube (Falcon; Becton-Dickinson, Franklin Lakes, NJ) containing 5 ml of BHI (Becton-Dickinson). Tubes were incubated aerobically with shaking (100 rpm) at 37 C for 24 hr. One milliliter of culture was removed for absorbance measurements at 600 nm using a Beckman DU-800 spectrophotometer (Beckman-Coulter, Fullerton, CA). Four milliliters of culture from each of two tubes were transferred to 100 ml BHI each in 125-ml Erlenmeyer flasks. Cultures were incubated with shaking (100 rpm) for 24 hr at 37 C and harvested by centrifugation at 10,000 × *g* at 5 C.

Protein extraction. Cell-free protein extracts were prepared as follows. The washed cells were resuspended in 20 mM 4-(2-hydroxyethyl) piperizane-1-ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 0.15 M sodium chloride at a concentration of 30 A_{600} units/ml and was sonicated for 5 min on ice using a microprobe at 50% duty cycle and 50% power (Model 250 cell disruptor; Branson, New Brunswick, NJ) and centrifuged at 15,000 × *g*. The cell-free sonicates were stored at −80 C until used.

Blood collection and hemolysis assays. Sheep and turkey red blood cells (RBCs) were obtained aseptically (by the veterinary staff of the National Animal Disease Center) from blood defibrinated by collection into sterile Erlenmeyer flasks containing 3-to-4-mm glass beads and two 2.5-cm (1-inch) lengths of coiled copper wire while gently swirling the flasks. Blood was poured aseptically through sterile gauze into sterile Erlenmeyer flasks and stored at 5 C and used within 3 days.

In vitro hemolysis assays were performed with both sheep and turkey RBCs. Briefly, sheep and turkey blood was collected aseptically in ethylenediaminetetraacetic acid–coated blood collection tubes (Becton-Dickinson) and centrifuged in 50-ml sterile conical tubes in a swinging bucket centrifuge at 400 × *g* for 10 min at 20 C. Erythrocytes were washed three times with phosphate-buffered saline (PBS; 50 mM sodium phosphate buffer and 0.15 M sodium chloride, pH 7.4) until the supernatant was colorless. A suspension of 1% RBCs (determined from the hematocrit volume) was made in 50 mM PBS containing 0.1% bovine serum albumin.

The hemolysis assay was performed as described previously (19) with some modifications. Briefly, proteins in 0.5-ml volumes containing 0.5, 1.5, and 2.25 mg protein/ml in 50 mM PBS were added to microfuge tubes containing 0.5 ml of RBC suspension. The tubes were gently inverted and incubated in a 37 C water bath. RBC lysis control (100%) was prepared by adding 0.5 ml of a 0.4% saponin (Sigma Chemical Co., St. Louis, MO) solution in water to 0.5 ml of RBC suspension. At specified time intervals, 0.2-ml aliquots were removed and centrifuged at

5000 × *g* for 1 min at room temperature. Supernatants were removed and deposited in Falcon 3595 (Costar, Cambridge, MA) microtiter plates. The absorbance was read at 540 nm using a model 250 microplate reader (SpectraMax 250; Molecular Dynamics, Sunnyvale, CA). Results were plotted using Excel (Microsoft, Redmond, WA).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Protein samples were denatured and chemically reduced using sample buffer from Invitrogen (Carlsbad, CA) containing 1 mM dithiothreitol and loaded onto 10-or-15-well 4%–20% gradient gels (Invitrogen). Samples were run according to the manufacturer's instructions, stained with Coomassie blue R250, and destained as described (30). Western blotting was performed using nitrocellulose membranes as described (48). For leukotoxin visualization, membranes were probed with a 1:10,000 dilution of anti-leukotoxin IgG monoclonal antibodies 2C9-1E8 (neutralizing) and 6A7-2E7 (nonneutralizing; R. E. Briggs *et al.*, unpubl. data) and prepared from *Mannheimia haemolytica* strain D153 serotype 1 using preparative SDS electrophoresis (Biorad Preparative Cell 491; Biorad, Hercules, CA). The neutralizing antibody was similar to MM601 (22). Unrelated IgG2b monoclonal antibody at 1:10,000 (obtained from P. Kapke, Iowa State University, and prepared to an unrelated recombinant bacterial protein) served as a control. Blots were incubated with the appropriate conjugates, alkaline phosphatase-conjugated anti-mouse IgG at 1:1000, or alkaline phosphatase-conjugated anti-turkey IgG at 1:1000. Color was developed for 10 min at room temperature using one 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablet per 10 ml (Sigma).

Isobaric tagging and relative and absolute quantitative (iTRAQ) analysis of *O. rhinotracheale* outer membrane proteins for leukotoxin peptides. Outer membrane proteins for iTRAQ analysis were prepared as follows. Four strains of *O. rhinotracheale* (P5883, P5884, P5886, ATTC 51463) were grown in 100 ml of BHI broth for 48 hr at 37 C with agitation (150 rpm), and centrifuged at 7500 × *g*. The cell pellet was washed with 0.1 M PBS, pH 7.2, and resuspended in a small volume (0.5 ml) of 2.5% NaCl in 20 mM HEPES, pH 7.4, and heated for 1 hr at 56 C to extract the outer membrane proteins (18). The extracted protein was dialyzed against 20 mM HEPES, pH 7.4. Protein concentration was determined using a bicinchoninic acid assay (Pierce Chemicals, Rockford, IL). Protein preparations were precipitated with ice-cold acetone (13), and 100 μg of each protein preparation was treated with one of four isobaric amino-modification reagents labeled with biotin (iTRAQ labels; Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. The protein preparations were digested with trypsin and mixed, and the peptides were separated by affinity chromatography on an avidin column as per manufacturer's directions. Samples were analyzed by liquid chromatography/mass spectrometry/mass spectrometry using the Q-Star XL-TOF quadrupole tandem mass spectrometer (Applied Biosystems) located at the Plant Science Institute, Iowa State University. Results were analyzed by using the Multi-Q software program (http://ms.iis.sinica.edu.tw/Multi-Q-Web/) (54).

RESULTS

Hemolytic activity on blood agar plates. Hemolytic activity was observed for most of the field strains after 48 hr at room temperature aerobically following an initial 48-hr incubation under 7.5% CO_2. Initial observations were recorded for isolate P5883 (serotype C, a chicken isolate), isolates P5884 and P5887 (both serotype A isolates from turkey), isolate P5886 (serotype B from turkey), and the reference strain ATTC 51463 (serotype A, a turkey isolate designated as the type strain for *O. rhinotracheale*) (47). For these strains and the subsequently tested additional field strains, α-hemolysis (greening of the blood agar) was often observed after 48 hr of incubation and upon removal of the plates from the CO_2 environment. Upon further incubation at room temperature, partial or complete hemolysis was observed, depending on the isolate tested.

Fig. 1. Hemolytic reaction of *O. rhinotracheale* field isolate P5932 on sheep blood agar. The isolate was incubated for 48 hr at 37 C, 7.5% CO_2, 15% relative humidity, followed by incubation aerobically at ambient temperature for 48 hr. Complete clearing under the confluent growth is evident as well as clearing under and surrounding the isolated colonies.

One field strain, isolate P5932, (Table 1; Fig. 1) (45) showed the earliest and most complete hemolysis of the isolates examined, followed by isolates P5883, P5884, and P5887, whereas the type strain ATCC 51463 exhibited a weak or no β-hemolytic reaction even after extended incubation aerobically at room temperature. In addition, isolate P5886 was nonhemolytic (no clearing). Some field isolates tested, whether they were able to grow in the presence of 2,2′-dipyridyl or not, showed some degree of α-hemolysis and β-hemolysis on sheep blood agar. Similar observations were recorded for hemolysis reactions on defibrinated turkey blood trypticase soy agar plates. Because sheep blood agar plates gave more consistent results (sheep RBCs remain intact and do not release nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate into the medium (2)) all experiments were subsequently performed using sheep blood agar plates.

Mass spectrometry identification of hemolysin orthologs. Because of the initial unexpected observations of hemolysis on blood agar, comparative proteomics using iTRAQ were employed to specifically compare labeled peptide fragments derived from the same protein (or proteins) expressed by different strains of *O. rhinotracheale*. Outer membrane extracts were prepared from four of the five characterized (45) isolates: P5883, P5884, P5886, and reference strain ATCC 51463 (Table 1). The results indicate that isolate P5884-labeled outer membrane proteins contained four peptides that were identical to the lktA of *M. haemolytica* and two peptides that were identical to *Actinobacillus actinomycetemcomitans* hemolysin (Table 2). An iTRAQ mass spectrum for peptide ^{600}KETKIIAK^{609} is shown in Fig. 2. The labeled extracts from isolates P5883, P5886, and ATCC 51463 showed lower relative intensities of the iTRAQ tag released from the respective hemolysin-like peptides peptide ^{600}KETKIIAK^{609}.

***In vitro* hemolytic and kinetic assays.** In order to confirm the observation of β-hemolytic activity observed on sheep blood agar plates and the iTRAQ results, *in vitro* hemolysis assays with both sheep and turkey RBCs were performed. The results shown in Fig. 2

Table 2. Hemolysin peptide homologues identified from iTRAQ analysis of *O. rhinotracheale* P5884 outer membrane proteins.

Peptide	Homology/accession no.	Score
^{221}KGLSGFDK^{229}	*M. haemolytica*/LKA16_PASHA	8.2
^{600}KTKETKIIAK^{609}	*M. haemolytica*/LKA16_PASHA	11.5
^{824}DLTFEKVNHHLVITNTKQEK^{844}	*M. haemolytica*/ LKA16_PASHA	8.73
^{896}IAQSELTK^{904}	*M. haemolytica*/ LKA16_PASHA	25.4
^{122}KHLSNSVGSTGNLTKAIDK^{139}	*A. actinomycetemcomitans*/Q43892	7.09
^{506}KKGEELAKHSDKFTK^{520}	*A. actinomycetemcomitans*/Q43892	15.9

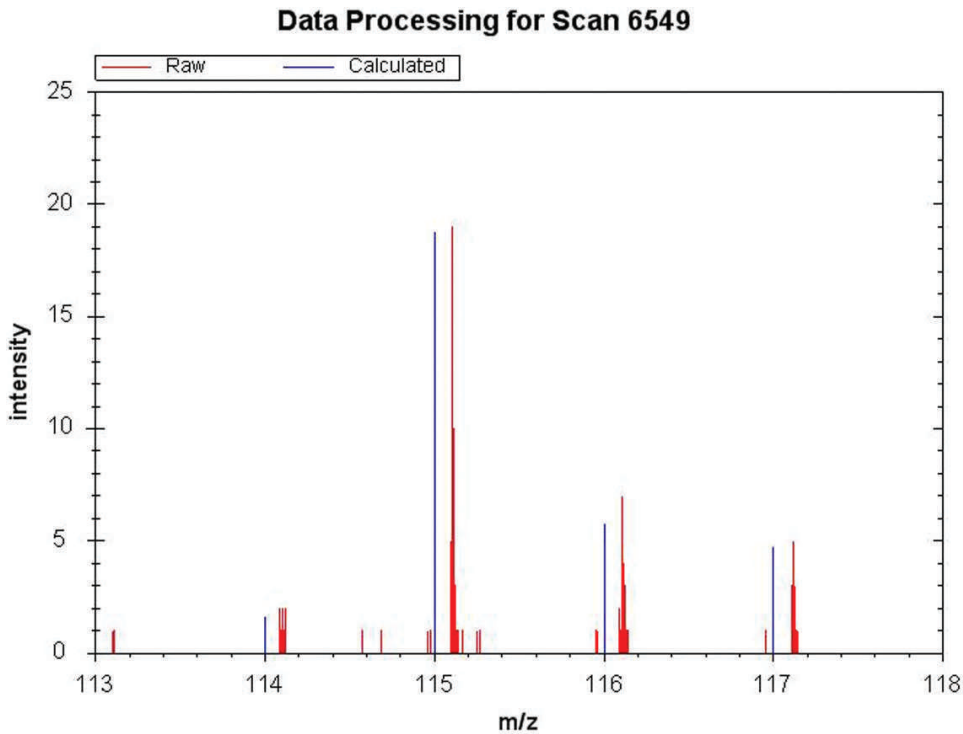

Fig. 2. iTRAQ results for the hemolysin-like activity of *O. rhinotracheale* strains. Relative intensities are shown for strains P5883 (tag 114), P5884 (tag 115), P5886 (tag 116), and ATCC 51463 (tag 117). Details are described under Materials and Methods. The red lines of the mass spectrum indicate the raw data; the blue lines show the normalized data.

depict a time-dependent assay using *O. rhinotracheale* cell-free sonicates obtained from isolates P5883, P5884, ATTC 51463, and P5932 and from sheep red blood cells. Results with turkey erythrocytes were comparable and are not shown. The highest hemolysin-like activity was observed from protein extracts prepared from field isolate P5932. At zero time, percentage of hemolysis of the buffer control, isolate P5883, and the reference strain ATCC 51463 were 11.7% compared to the positive control of saponin (set at 100% hemolysis); isolates P5884 and P5932 exhibited 29% and 71% hemolysis, respectively, compared to the positive control (Fig. 3). We observed that hemolysis takes place within the first minute of incubation at 37 C. Therefore, for subsequent assays, the first sample was removed at 30 sec, and at 2-to-3-min intervals thereafter. The highest hemolytic activity was observed for protein extracts from isolate P5932. Therefore protein extracts from this isolate were used in subsequent assays for concentration-dependent and kinetic analyses. Fig. 4 depicts the concentration-dependent hemolytic assay with the cell-free sonicates from strain P5932. Initial rates of hemolysis measured at the 30-sec time point were directly proportional to the protein concentration of the cell-free sonicates.

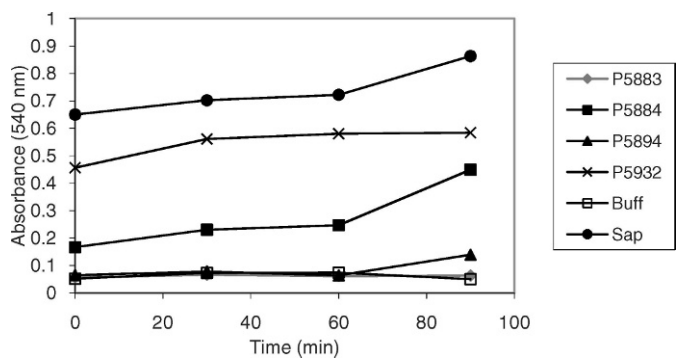

Fig. 3. Time-dependent hemolytic activity of *O. rhinotracheale* protein extracts. Cell-free sonicates were assayed at a final protein concentration of 1 mg/ml using the *in vitro* hemolytic assay with 0.5% sheep RBCs. Absorbance of the assay supernatants were measured after centrifugation at 540 nm. P5883, P5884, P5894 (ATCC 51463), and P5932 are the cell-free sonicates; Buff indicates the buffer control sample and Sap indicates the positive control sample of RBCs lysed with saponin.

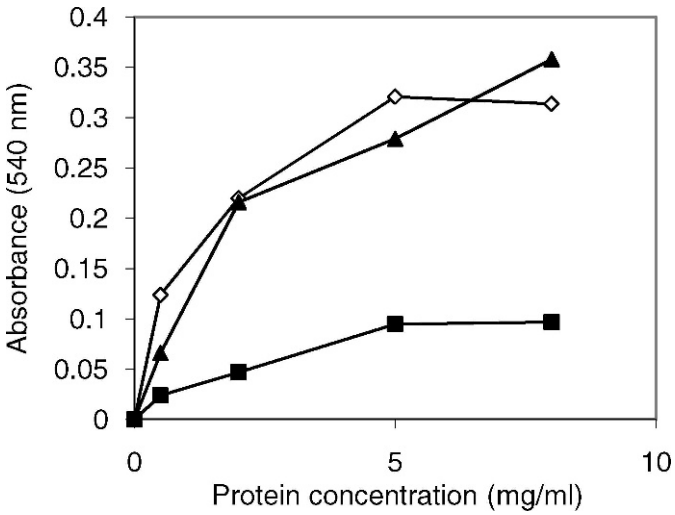

Fig. 4. Concentration-dependent hemolytic activity of *O. rhinotracheale* strain P5932 hemolysis-like activity. Plot of absorbance at 540 nm *vs.* protein concentration of the cell-free sonicates of strain P5932 are shown at protein concentrations of 0.5 (■), 1.5 (▲), and 2.25 (◇) mg/ml in the assay.

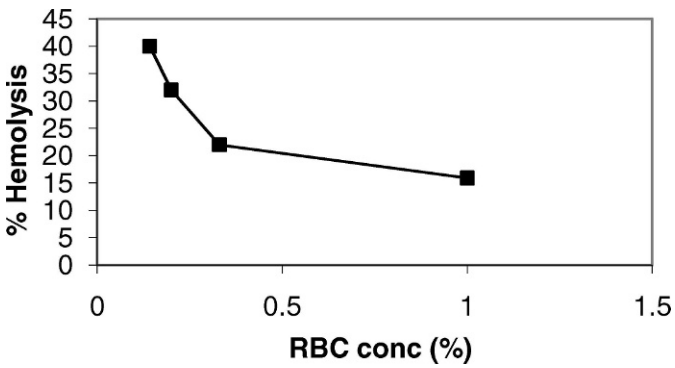

Fig. 5. Plot of percentage of hemolysis *vs.* RBC concentration. Percentage of hemolysis decreases with increasing RBC concentration suggesting that the hemolytic activity is a pore-forming protein. Protein concentration was at 2.5 mg/ml in the assay.

Kinetic analyses of the substrate-dependent data, the RBC concentration in the assay, (Fig. 5) suggests that the hemolytic activity was due to a pore-forming ability of the protein and was not due to enzyme action on the RBC membrane (39), as the percentage of hemolysis decreased with increasing RBC concentration. If the hemolytic activity were an enzyme, percentage of hemolysis would have increased with increasing RBC concentration.

1-Dimensional SDS-PAGE and western blotting of a hemolysin-like protein. Because iTRAQ comparative experiments for global analysis of the *O. rhinotracheale* proteome identified proteins with sequence identity toward four peptide sequences of lktA of *M. haemolytica*, western blotting was performed using highly specific IgG monoclonal antibodies prepared to lktA (Fig. 6). Numerous proteins appeared on the Coomassie-stained gel (Fig. 6A), but only three proteins were visualized on the western blot using the neutralizing monoclonal antibody, 2C9, and the nonneutralizing antibody, 6A7 (Figs. 6B and 6C, respectively). The weakly staining bands corresponded to proteins at 181 and 92 kDa and the strongly staining band corresponded to a protein (or proteins) at 56 kDa.

To rule out potential reactivity of the monoclonal IgG with a putative IgG binding protein, an unrelated IgG2b monoclonal antibody prepared to an unrelated recombinant protein expressed in *E. coli* (Paul Kapke, Iowa State University) was used in a separate western blot experiment (results not shown). This monoclonal also reacted with a protein corresponding to a band at 56 kDa, but at a lower intensity than the monoclonal antibodies to lktA. The unrelated IgG2b monoclonal antibody did not react with the 181- or 92-kDa proteins on the western blot. The difference in intensities could be due to variation in titer of the specific and nonspecific monoclonal antibodies or due to the fact that less putative IgG-binding protein was present in the band. It is possible that staining of the 56-kDa protein (or proteins) resulted because of binding to an as yet unidentified IgG-binding protein in addition to an unidentified hemolysin-like protein of low molecular weight.

DISCUSSION

In 1994, an *O. rhinotracheale* isolate from clinical samples from turkeys was characterized by Vandamme and colleagues (49). This isolate of *O. rhinotracheale* was designated as the reference strain for

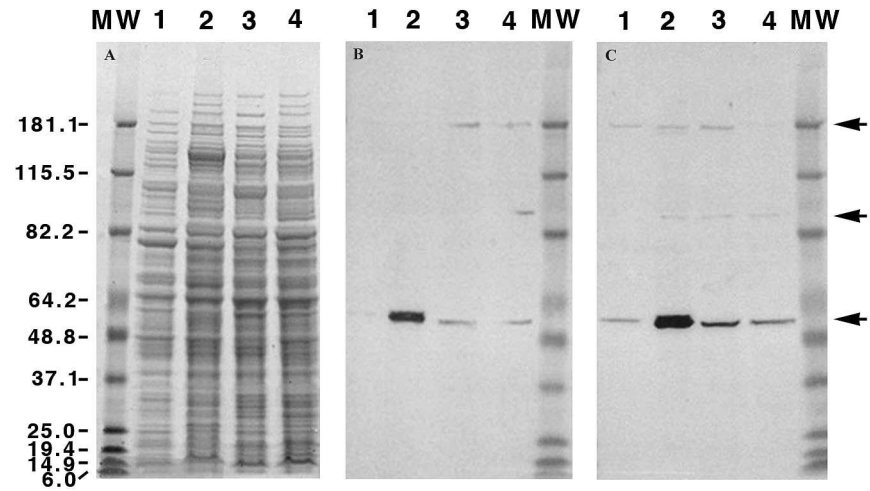

Fig. 6. SDS-PAGE and western blot of cell-free sonicates from *O. rhinotracheale* strains. (A) SDS-PAGE of cell-free lysates from P5883 (lane 1), P5884 (lane 2), ATCC 51463 (lane 3), P5932 (lane 4). (B) Western blot developed with anti-lktA monoclonal antibody 2C9-IE8. (C) Western blot with anti-lktA monoclonal antibody 6A7-2E7. For B and C, lanes are the same as indicated in A. MW, molecular weight markers in kilodaltons. Arrows indicate the bands at 181, 92, and 56 kDa.

Table 3. β-Hemolytic activity on sheep blood agar plates of the *O. rhinotracheale* strains used in this study.

Strain no.	Reaction after 48 hr, 37 C, CO_2[A]	Reaction after 48 hr, room temp, air[B]
Original strains used		
P5883	1 mm, clearing initiated	Complete clearing
P5884	1 mm, clearing initiated	Complete clearing
P5886	1 mm, no clearing	No clearing
P5887	1 mm, clearing initiated	Complete clearing
ATCC 51463	1 mm, no clearing	No clearing
Isolates capable of growth under iron-restricted conditions		
P5902	1 mm, clearing initiated	Complete clearing
P5917	1 mm, clearing initiated	Complete clearing
P5921	1 mm, clearing initiated	Complete clearing
P5932	1 mm, clearing initiated	Complete clearing
P5938	1 mm, no clearing	No clearing
P5945	1 mm, no clearing	No clearing
P5952	2 mm, no clearing	No clearing
P5967	1 mm, clearing initiated	Complete clearing
Isolates incapable of growth under iron-restricted conditions		
P5907	1 mm, clearing initiated	Complete clearing
P5908	1 mm, clearing initiated	Complete clearing
P5909	0.5 mm, clearing initiated	Complete clearing
P5913	1 mm, clearing initiated	Complete clearing
P5926	1 mm, no clearing	No clearing
P5936	1 mm, no clearing	No clearing
P5940	1 mm, clearing initiated	Complete clearing
P5963	2 mm, clearing initiated	Complete clearing
P5970	1 mm, clearing initiated	Complete clearing

[A]Reaction on blood agar after 48 hr of incubation under CO_2; sizes of colonies are indicated; clearing of blood agar observed under confluent growth, but not yet under the individual colonies.

[B]Reaction on blood agar after 48 hr of incubation at room temperature in air; clearing of blood agar observed under most individual colonies.

O. rhinotracheale (ATCC 51463) and was described as a non–β-hemolytic species using sheep blood agar. The isolates used for the current study were North American clinical isolates obtained from the culture collection of K. V. Nagaraja (University of Minnesota, St. Paul, MN; Table 1). Interestingly, the majority of the North American field isolates of *O. rhinotracheale* showed β-hemolytic reactions on sheep blood agar when the incubation period was extended beyond 48 hr under CO_2 with further incubation at ambient temperature for an additional 48 hr (Table 3; Fig. 1). This observation is not unusual for certain microbes and incubation under aerobic conditions and has been done to identify oxygen-stable hemolysins (53). One explanation for the observed difference between the data reported here and those reported by Vandamme *et al.* (49) may be the acquisition of the hemolysin/cytolysin gene by horizontal transfer. Alternatively, it is possible that the gene encoding the hemolysin/cytolysin is not expressed in the type strain described by Vandamme *et al.* (49). Genetic analysis of these strains, although not within the scope of this study, may resolve this issue. Furthermore, it is unknown if the hemolytic-like activity is related to virulence of *O. rhinotracheale*. The hemolytic reactions on sheep blood agar reported here were also observed on turkey blood agar plates. Although of interest, no other cytolytic activities or cell substrates were examined in this study. Furthermore, this study did not directly associate the hemolytic activity with any one of the three proteins identified by western blotting with the lktA-specific monoclonal antibody. It would be of interest to compare the relative virulence of the hemolytic and nonhemolytic phenotypes in the turkey model described by Sprenger *et al.* (43).

In order to identify the hemolytic activity of *O. rhinotracheale* strains, secreted proteins, outer membrane proteins, and whole-cell lysates were examined by iTRAQ mass spectrometry methodology. iTRAQ methodology is the technique of choice for comparative global analysis of proteins expressed by various strains. In this particular study, the data of the iTRAQ experiments were searched for potential hemolysins to corroborate the hemolytic reactions observed on blood agar. Indeed, analysis of iTRAQ experiments identified peptides from *O. rhinotracheale* protein extracts that were identical to peptides of lktA and peptides of *Actinobacillus actinoacetemcomitans* as shown in Fig. 2 and Table 2.

Kinetically, three different types of hemolytic (cytolytic) mechanisms have been described based on the analysis of concentration-dependent *in vitro* assays (4,9). These types are 1) porins, 2) enzymes, and 3) surfactants. Based on kinetic analysis of the hemolytic activity of isolate P5932, we determined that the hemolytic activity was a pore-forming molecule rather than an enzyme molecule (47). However, because the kinetic analysis cannot distinguish between a pore-former and a molecule with surfactant properties, complete DNA and protein sequence information is needed to make this determination. Attempts to clone a hemolysin-like gene with degenerative primers based on the peptides identified by mass spectrometry and southern blotting experiments using probes based on *M. haemolytica* leukotoxin were not successful (F. Tatum, B. Briggs, and L. B. Tabatabai, unpubl. data). Genomic sequencing will eventually provide the results of the nature of the hemolysin/cytolysin molecule.

Western blots prepared from 1-D gels of *O. rhinotracheale* whole-cell lysates and treated with the monoclonal antibodies 2C9-1E8 and 6A7-2E7 to *M. haemolytica* leukotoxin showed three bands at 181, 92, and 56 kDa. A search of the literature for hemolytic molecules indicated hemolysin-like molecules with molecular masses of approximately 180,000, 102,000, and 56,000. These molecules include cytolysins (4,29,31,32,47), Zn-metalloprotease (11), and certain amino-peptidases (5) of both gram-negative and gram-positive microbes. Interestingly, a western blot treated with an

unrelated IgG2b monoclonal antibody indicated that some reactivity occurred with a band at 56 kDa, and may be due to an as yet unidentified IgG-binding protein, which is expressed by many gram-negative pathogens.

In summary, this is the first report that North American field isolates of *O. rhinotracheale* express a hemolysin-like protein present in the whole cell lysate. This hemolytic activity may contribute to the overall virulence of *O. rhinotracheale* (6,40,41,42,50).

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